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Anaerobic Decomposition of Switchgrass by Tropical Soil-Derived Feedstock-Adapted Consortia

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ABSTRACT Tropical forest soils decompose litter rapidly with frequent episodes of anoxic conditions, making it likely that bacteria using alternate terminal electron acceptors (TEAs) play a large role in decomposition. This makes these soils useful templates for improving biofuel production. To investigate how TEAs affect decomposition, we cultivated feedstock-adapted consortia (FACs) derived from two tropical forest soils collected from the ends of a rainfall gradient: organic matter-rich tropical cloud forest (CF) soils, which experience sustained low redox, and iron-rich tropical rain forest (RF) soils, which experience rapidly fluctuating redox. Communities were anaerobically passed through three transfers of 10 weeks each with switchgrass as a sole carbon (C) source; FACs were then amended with nitrate, sulfate, or iron oxide. C mineralization and cellulase activities were higher in CF-FACs than in RF-FACs. Pyrosequencing of the small-subunit rRNA revealed members of the *Firmicutes*, *Bacteroidetes*, and *Alphaproteobacteria* as dominant. RF- and CF-FAC communities were not different in microbial diversity or biomass. The RF-FACs, derived from fluctuating redox soils, were the most responsive to the addition of TEAs, while the CF-FACs were overall more efficient and productive, both on a per-gram switchgrass and a per-cell biomass basis. These results suggest that decomposing microbial communities in fluctuating redox environments are adapted to the presence of a diversity of TEAs and ready to take advantage of them. More importantly, these data highlight the role of local environmental conditions in shaping microbial community function that may be separate from phylogenetic structure.

IMPORTANCE After multiple transfers, we established microbial consortia derived from two tropical forest soils with different native redox conditions. Communities derived from the rapidly fluctuating redox environment maintained a capacity to use added terminal electron acceptors (TEAs) after multiple transfers, though they were not present during the enrichment. Communities derived from lower-redox soils were not responsive to TEA addition but were much more efficient at switchgrass decomposition. Though the communities were different, diversity was not, and both were dominated by many of the same species of clostridia. This reflects the inadequacy of rRNA for determining the function of microbial communities, in this case the retained ability to utilize TEAs that were not part of the selective growth conditions. More importantly, this suggests that microbial community function is shaped by life history, where environmental factors produce heritable traits through natural selection over time, creating variation in the community, a phenomenon not well documented for microbes.

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Liquid biofuels derived from nonfood crops, such as switchgrass, are a promising replacement for nonrenewable petroleum-based fuels. The U.S. Department of Energy has identified cellulosic biofuels as integral to meeting our current and future energy needs, partly due to a large renewable reservoir of plant biomass (1, 2). Although corn- and sugarcane-derived ethanol production is already under way on a significant commercial scale, cellulosic ethanol production is inefficient and costly; enzymes derived from fungi are expensive to produce and still require feedstocks to be pretreated before fermentation. Alternative biofuels do not compete with food sources, require less energy input, and produce fewer greenhouse gases than corn ethanol (3,

4). Biofuel production from switchgrass and other perennial grasses has the potential to provide more than one-third of the energy for transportation fuels domestically (1, 5) and produce 540% more renewable energy than that consumed for production, with 94% lower greenhouse gas emissions than for gasoline (6). While switchgrass presents a viable solution to improving biofuel cost and energy efficiency, conversion of lignocellulose to sugars stands to be improved through microbial processing.

Tropical forest ecosystems have very high litter and root decomposition rates (7, 8), and these soils have demonstrated the potential to efficiently deconstruct switchgrass into simple compounds (9). High rates of decomposition are fueled by a high

microbial biomass and consistent warm and humid conditions (7, 10). It has been hypothesized that iron cycling fuels considerable decomposition; tropical forest soils experience frequent episodes of anoxic conditions (11, 12), so direct or indirect iron reduction could be an avenue of growth, with biotic or abiotic regeneration of this terminal electron acceptor under oxic conditions. Fluctuating redox potential conditions create an environment where anaerobic and facultative bacterial metabolisms play a large role in leaf litter decomposition.

Under anaerobic conditions, a complex microbial community comprised of fermenters, anaerobic respirers using a range of terminal electron acceptors (TEAs), and syntrophic and acetoclastic methanogens likely contributes to decomposition (13, 14). This is distinct from temperate systems where fungi dominate litter decomposition through the release of phenol oxidase and peroxidase, enzymes that require oxygen (O_2) to create free radicals (15). Anaerobic bacteria capable of depolymerizing lignin use different enzymes, such as the beta-aryl etherase produced by *Sphingomonas paucimobilis* (16). There is a large unexplored diversity of bacteria involved in anaerobic decomposition, likely representing novel approaches to lignin depolymerization in particular that address the challenges of biomass conversion. This would be advantageous for large-scale plant biomass conversion, since the production and metabolic engineering of bacteria are easier than those for fungi (17). Understanding the mechanisms of decomposition in these naturally occurring microbial communities will improve development of lignocellulosic biofuels.

We explored patterns in enzymatic activity and microbial diversity between soil types at the extreme ends of an established elevation, rainfall, and soil redox gradient in the Luquillo Experimental Forest, Puerto Rico (12). The two sites were a low-elevation rain forest site with rapidly fluctuating redox (RF) and a high-elevation cloud forest site with low and slowly fluctuating redox (CF). Decomposition is fast at both sites, but degradation of leaf litter *in situ* was nearly an order of magnitude faster in the lower-elevation sites than in the high-elevation sites (7; W. L. Silver, R. Ostertag and A. Thompson, unpublished observation). Both sites have measurable methane production, though net methane production is considerably higher at the upper-elevation site (18). Both soils are rich in poorly crystalline Fe oxide minerals, which are insoluble but somewhat unstable and available for microbial reduction. In soils that boast up to 10^{11} cells per g, iron reducers are found at up to 10^9 cells per g soil, or 1% of total microbial abundance (10). Iron cycling, which itself is driven both by high Fe abundance and frequent fluctuations in soil redox potential (12, 19, 20), can oxidize C equivalent to 44% of annual litter production (7). Dissimilatory Fe(III)-reducing bacteria compete effectively for limited C with methanogens (21), and there is excellent correlation between CO_2 production and Fe(II) production (10). These soils also cycle N and S rapidly (22–24), which suggests that the identity and abundance of TEAs may have a significant impact on the microbial community and their associated decomposition enzymes.

To understand the role of different TEAs in decomposition and investigate the possibility for exogenous TEAs to change either the microbial communities or the decomposition rates under controlled laboratory conditions, we set up anaerobic feedstock-adapted consortia (FAC) derived from the rain forest site (RF) and cloud forest site (CF). After three transfers, the richness of consortia was about one-tenth that of the original soil inocula, as previ-

ously described (9). Once these three replicate consortia were established on switchgrass under anaerobic conditions, we used them to seed new consortia (each with three biological replicates) and measured switchgrass decomposition under fermentative conditions (switchgrass alone) and also with potential denitrification (nitrate added), Fe reduction [iron(III) added], and sulfate reduction (sulfate added). We then examined how exogenously added TEAs affect switchgrass C mineralization as well as microbial community function and structure.

RESULTS

Microbial activity. To estimate the C mineralization associated with switchgrass degradation, we measured the amount of CO_2 and CH_4 emitted; controls with no switchgrass added had no measurable emissions of CO_2 or CH_4 . Carbon mineralization was significantly higher for the cloud forest (CF)-derived FACs than for the rain forest (RF)-derived FACs, regardless of TEAs added, in terms of cumulative CO_2 production ($P < 0.01$) and CH_4 production ($P < 0.001$) per gram switchgrass added. TEA addition had significant effects on CO_2 in the RF ($P < 0.0001$) and CF ($P < 0.0001$) and on CH_4 in the RF ($P < 0.05$) and CF ($P < 0.0001$) FACs (Fig. 1). The nitrate amendment had the highest CO_2 production in the RF-FACs but the lowest CO_2 production in CF-FACs. Iron significantly increased the CO_2 above the background level in the RF-FACs. CO_2 and CH_4 production was suppressed following TEA amendment in the CF-FACs.

Changes in the concentrations of NO_3^- , NO_2^- , Fe(II), and SO_4^{2-} were measured at the beginning and end of the incubation to estimate redox sensitivity of the microbial communities (Table 1). Net reduction of added NO_3^- to NO_2^- was detected in the RF-FACs but not the CF-FACs. Net Fe reduction occurred in both the RF- and CF-FACs that received Fe oxide, as evidenced by the generation of high concentrations of Fe(II) at the end of the incubation. A small but significant amount of net sulfate was reduced in the sulfate-amended RF-FACs.

Beta-galactosidase and cellobiohydrolase activities were significantly higher in CF-derived FACs than in RF-FACs (4-methylumbelliferyl β -D-glucopyranoside (MUB) [BG], $P < 0.001$; β -D-cellobioside-MUB [CBH], $P < 0.05$), but there was no difference in activity between sites for N-acetylglucosidase or xylosidase activities. There were significant treatment effects in RF-FACs for BG ($P < 0.05$), CBH ($P < 0.01$), N-acetylglucosamine-MUB (NAG) ($P < 0.0001$), and β -D-xylopyranoside-MUB (XYL) ($P < 0.0001$). The scale of the effect of TEAs on enzyme activities was relatively small, with the largest differences being 31% (BG), 56% (CBH), 234% (NAG), and 61% (XYL). There were weakly significant treatment effects in CF-FACs for NAG ($P = 0.081$) and XYL ($P = 0.078$) and no effect for BG or CBH (Fig. 2).

Microbial community growth and structure. Higher C mineralization rates observed in CF-FACs than in RF-FACs did not translate to cell abundance or biomass. There was no significant difference between inocula based on total lipids extracted or direct counts, whether comparing final cell counts or fold increase in cell growth (final count divided by initial count) (see Fig. S1 in the supplemental material). There was a significant treatment effect of TEAs on cell growth in RF-FACs ($P < 0.0001$) but not in CF-FACs. Among the RF-FACs, the sulfate-amended incubations had the most growth, followed by the switchgrass and iron-amended incubations, with the nitrate-amended incubations having the lowest growth (see Fig. S1A). When biomass was measured by

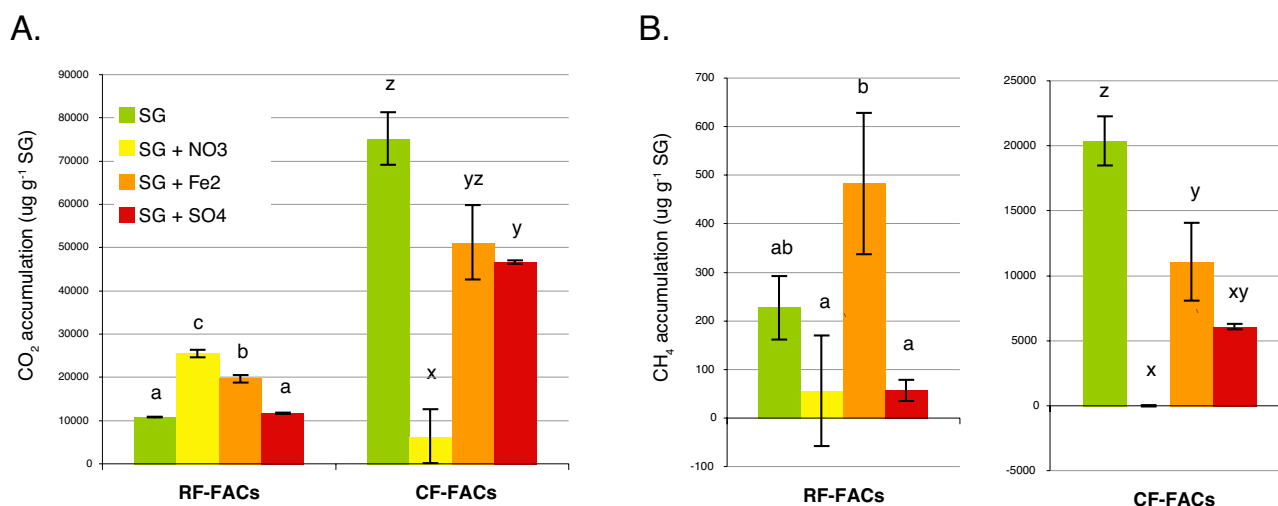


FIG 1 Net CO₂ (A) or CH₄ (B), expressed as $\mu\text{g C}$ per gram switchgrass accumulated in the FACs over the course of the incubation. Values shown are means plus or minus standard errors ($n = 3$). Compared to the RF-FACs, the CF-FACs had significantly more CO₂ ($P < 0.01$) and CH₄ emitted ($P < 0.001$). Within each FAC, the treatment effect was assessed; treatment had a significant effect on FACs in all cases. Lowercase letters denote treatments that are statistically indistinguishable within each set ($n = 3$), as determined by Tukey's HSD test at a cutoff of $P < 0.05$.

lipids extracted, there was no treatment effect for either inoculum type (see Fig. S1B).

Pyrosequencing resulted in 1,310 unique taxa resolved at 100% nucleotide identity. There were no differences between RF- and CF-FAC inocula in richness or diversity (by Shannon's H or Simpson's D index). Treatment significantly affected richness and diversity, where the nitrate treatment was lower in diversity compared to the baseline switchgrass, iron-amended, and sulfate-amended FACs; these data are based on pooled RF- and CF-FAC diversity estimates (Table 2). Nitrate treatments lost taxa primarily from the *Proteobacteria* and rare species in the RF-FACs (see Table S2 in the supplemental material) and from members of the *Proteobacteria*, *Bacteroidetes*, *Methanobacteria*, and *Acidobacteria* in the CF-FACs (see Table S3 in the supplemental material). Ordination of microbial communities using phylogenetic distances revealed significant differences in microbial community profiles based on inoculum ($A = 0.028$ [see Materials and Methods]; $P < 0.05$) and treatment ($A = 0.042$; $P = 0.054$) (Fig. 3). Joint plots for values of microbial community activity showed that C mineraliza-

tion, enzyme activities, and cell counts were all strongly and significantly correlated to phylogenetic community structure ($P < 0.05$).

Microbial community profile by phospholipid fatty acid analysis (PLFA) showed separation of treatments, though no separation by inoculum (see Fig. S1C in the supplemental material). The 18:0 lipid marker for general biomass was higher in baseline switchgrass and nitrate-amended FACs than in iron and sulfate-amended FACs (see Fig. S2 and Table S1), which may be due to residual lipids from the switchgrass. Of the four lipid markers for Gram-negative bacteria that were significantly affected by TEA treatment, one (17:1w9c) was highest in iron compared to nitrate, while the rest were highest in switchgrass only compared to iron (see Fig. S2 and Table S1). The stress biomarker lipid 16:1w7t was significantly elevated in unamended FACs. Total lipids did not vary significantly by TEA treatment.

Dominant taxa were defined as taxa with a relative abundance of at least 2% in at least one sample; 23 taxa in RF-FACs and 14 taxa in CF-FACs fit this criterion. Most dominant taxa were members of the *Clostridia* (phylum *Firmicutes*); 15 of the RF-FAC dominants and 8 of the CF-FAC dominant taxa were members of the *Clostridia* (Fig. 4). Taxa in cluster 53 (*Rhizobiales*), clusters 6 and 24 (*Clostridia*), and cluster 42 (*Bacteroidetes*) were all enriched in iron-amended RF-FACs. The switchgrass-only and Fe-amended CF-FACs had the highest relative abundances of the archaeal methanogens *Methanosarcina* and *Methanobacterium* and also the highest net methane production. The SO₄-amended CF-FACs contained *Methanobacterium* species but *Methanosarcina* species as dominant organisms, suggesting that *Methanobacterium* was primarily responsible for methane production in these microcosms. Among the few eukaryotes grown in these samples, stramenopiles were enriched in sulfate-amended CF-FACs and were dominant ($>2\%$ relative abundance) in one replicate (see Table S6 in the supplemental material). Overall, the three replicate microcosms were surprisingly similar considering that they had been separately transferred by 10% serial dilution into fresh media three times.

TABLE 1 Concentrations of added TEAs at the beginning and end of FAC lab incubations

Process and consortia	Added TEA	Concn (mM) of TEA		P value ^a
		T _{initial}	T _{final}	
Denitrification				
Rain forest FACs	NO ₃ [−]	11.4 ± 0.07	0.01 ± 0.01	***
	NO ₂ [−]	0.01 ± 0.01	3.66 ± 0.44	**
Cloud forest FACs	NO ₃ [−]	11.6 ± 0.13	10.08 ± 1.59	
	NO ₂ [−]	0.03 ± 0	0.02 ± 0	
Iron reduction				
Rain forest FACs	Fe(II)	0.44 ± 0.06	6.87 ± 0.07	***
Cloud forest FACs	Fe(II)	0.71 ± 0.06	6.4 ± 0.26	***
Sulfate reduction				
Rain forest FACs	SO ₄ [−]	7.89 ± 0.32	6.15 ± 0.3	*
Cloud forest FACs	SO ₄ [−]	7.85 ± 0.15	7.91 ± 0.13	

^a ANOVAs were evaluated at P value cutoffs of <0.05 (*), <0.01 (**), and <0.001 (***) ($n = 6$).

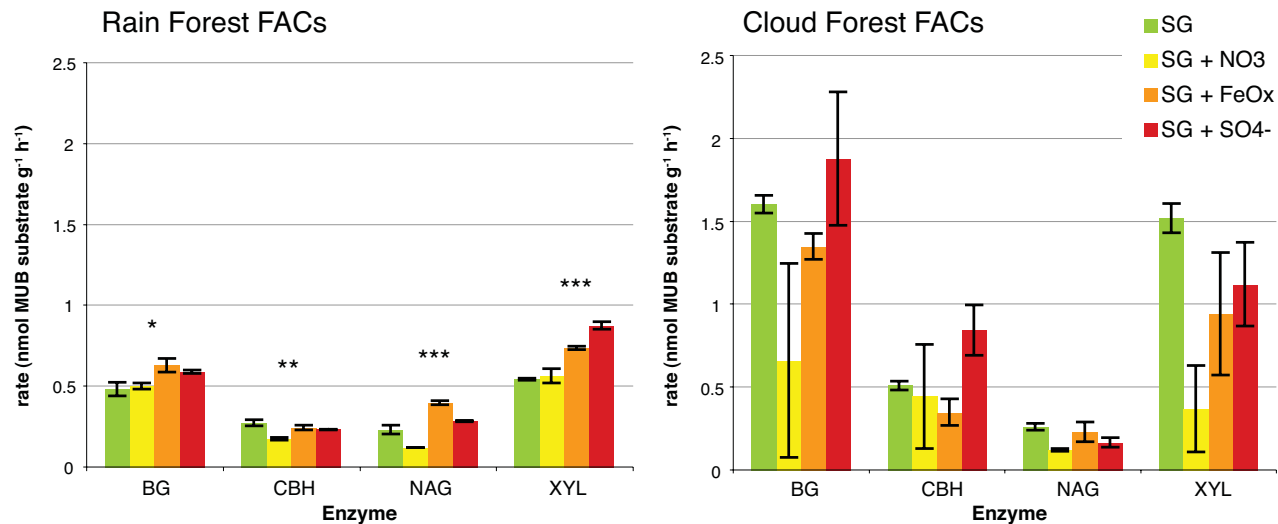


FIG 2 Enzyme activities for beta-glucosidase (BG), cellobiohydrolase (CBH), *N*-acetyl glucosaminidase (NAG), and xylosidase (XYL) are shown for the RF-FACs (left) and CF-FACs (right), expressed as nmol substrate converted per gram of biomass per hour. Values shown are means plus or minus standard errors ($n = 3$), and ANOVAs were performed to test the null hypothesis of no difference between TEA treatments within an enzyme activity rate, with associated P values shown as “*” ($P < 0.05$), “**” ($P < 0.01$) and “***” ($P < 0.001$).

DISCUSSION

Through cultivation of feedstock-adapted consortia (FACs) derived from tropical rain forest (RF) and cloud forest (CF) soils, we found that the RF-FACs were able to make use of a broader suite of TEAs than CF-FACs, while the CF-FACs were more productive than RF-FACs. In each case, productivity was indicated by respiration, cell biomass, and enzyme activity during deconstruction of switchgrass under static anoxic conditions. The RF-FACs mostly responded positively to added TEAs. In comparison, the CF-FACs were unresponsive to TEAs and had overall higher CO₂ and CH₄ accumulation and higher enzyme activities per gram switchgrass. This is particularly surprising given the more rapid decomposition rates of the rain forest site under field conditions (Silver et al., unpublished). The reduction of Fe(III), SO₄²⁻, and NO₃⁻ by tropical soil communities has the potential to drive considerable C mineralization (10, 22, 24, 25), so these experiments were designed to explore the extent to which anaerobic switchgrass decomposition would be affected by TEA additions in culture. We suggest that a combination of the differential abundances of specific organisms (such as dominance of acetate-utilizing *Methanosarcina* in the CF-FACs) and the life history of shared microbial populations were responsible for the differences in ability to use different TEAs and in C mineralization efficiency between the two tropical forest soil inocula.

The cloud forest feedstock-adapted consortia (CF-FACs) were more efficient at decomposing switchgrass: higher enzyme activity and rates of C mineralization of CF-FACs were accompanied by similar numbers of cells and amounts of biomass compared to findings for RF-FACs. The greater efficiency of biomass conversion of the CF-FACs could be explained by the different life histories of soil microbes from the tropical cloud forest compared to those from the rain forest. The higher-elevation cloud forest soils experience frequent low-redox conditions, while the lower-elevation rain forest soils experience higher overall redox potential and so are adapted to more-frequent O₂ availability. In general, tropical forest soils are likely to be adapted to fluctuating redox; compared to static oxic or anoxic conditions, fluctuating redox resulted in the highest number of active microbes (25, 26). Based on these studies, we would hypothesize that the higher-elevation, lower-O₂ soils should have lower field decomposition rates; indeed, this has been shown under field conditions (7). However, under anoxic laboratory conditions the lower-redox CF-derived FACs performed better than the RF-derived FACs. The increased C mineralization of CF-FACs over that of RF-FACs is apparently not due to increased biomass, since these two inocula were indistinguishable by direct counts and by lipid biomass, suggesting that an equivalent number or mass of microbes was able to more efficiently deconstruct the feedstock. Either the planktonic nature of

TABLE 2 Diversity of FACs by TEA treatment, calculated as overall richness, Shannon’s diversity index (H), Simpson’s index (D), and inverse Simpson’s index (invD)

Statistic	Value for treatment ^a				P value ^b
	SG	SG + NO ₃ ⁻	SG + FeOx	SG + SO ₄ ²⁻	
Richness	172.2 ± 39.1 b	54.8 ± 57.1 a	148.8 ± 105.6 ab	123.8 ± 29.9 ab	*
H	2.46 ± 0.27 b	0.75 ± 0.73 a	2.05 ± 1.04 b	2.23 ± 0.33 b	**
D	0.84 ± 0.04 b	0.33 ± 0.31 a	0.71 ± 0.35 b	0.83 ± 0.05 b	**
invD	6.45 ± 1.51 b	1.83 ± 0.96 a	5.94 ± 2.89 b	6.14 ± 1.71 b	**

^a Lowercase letters denote different levels of significance as determined by Tukey’s HSD test at $P < 0.05$. SG, switchgrass; FeOx, iron oxide.

^b ANOVAs were evaluated at P value cutoffs of <0.05 (*) and <0.01 (**). ($n = 6$).

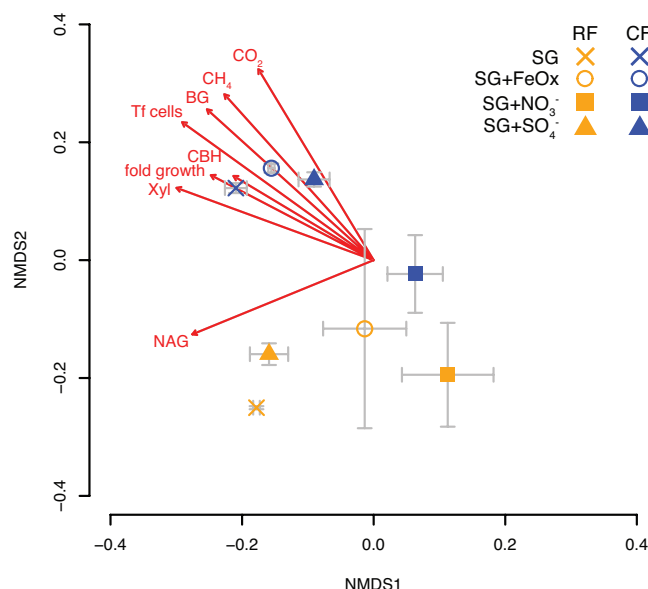


FIG 3 Microbial community profile based on unifracs distance calculated for pyrotag-sequenced SSU rRNA genes. A community profile model was made using ordination by nonmetric multidimensional scaling (NMDS) based on a Bray-Curtis distance metric. Community members shown are means of three replicates, with error bars denoting standard errors of the means ($n = 3$). Joint plots display vectors showing direction and strength (as length) of positive correlation of the community with each of the listed traits; only significant correlations ($P < 0.05$) are plotted.

the lab incubations, the static anaerobic conditions, or some property of the plant biomass added was favorable to the CF-FACs compared to the RF-FACs.

Carbon mineralization was inhibited by TEA addition in the CF-FACs. It is possible that the absence of these alternative electron acceptors during the adaptation of microbial consortia to anaerobic switchgrass decomposition could have resulted in a decreased ability to reduce NO₃⁻, Fe(III), and SO₄⁻; further research could address this point. In contrast, we observed considerable plasticity in the metabolism of the RF-FACs, which retained facultative metabolic capacities for coupling NO₃⁻ and SO₄⁻ reduction to decomposition. It is possible that the presence of a strong oxidizer, such as NO₃⁻, was inhibitory to the cloud forest-derived communities, which would have been accustomed to low redox. These CF-FAC communities were making 10 times more CH₄ than the RF-FACs before the addition of NO₃⁻ depressed them; this could be evidence of low-level NO₃⁻ utilization below detection of the chemical analysis performed here. It is possible that low levels of nitrite or sulfite were produced in the CF-FACs and that these were enough to inhibit methanogenesis (13, 27). That both FACs retained the ability to utilize Fe(III) as an alternative TEA is a reflection of the prominence of reactive Fe species in these soils and its utility in microbial growth and activity.

Organisms with facultative metabolic capabilities seemed to be preserved throughout the transfers in the RF-FACs but not the CF-FACs, despite the fact that all consortia were adapted without the addition of exogenous TEAs. This was evident in the SO₄⁻-amended FACs, where addition caused a significant increase in cell growth, as well as a significant increase in xylosidase enzyme activity. Compared to findings for other TEA treatments, the SO₄⁻-amended RF-FACs had increased numbers of *Desulfovibrio*

bacteria, which are known SO₄⁻ reducers (28) with innate tolerance for O₂ that would aid in survival in fluctuating-redox soils (29). *Desulfovibrio* was also enriched in the Fe(III)-amended RF-FACs, though to a lesser extent than in the SO₄⁻-amended FACs. This suggests the possibility of Fe(III)-reducing *Desulfovibrio*, though members of the genera *Clostridium* and *Aeromonas* were also present and were likely reducing Fe(III) (30, 31). Siderophores, humics, or other electron shuttles may permit a range of microbes to indirectly reduce Fe(III), producing Fe(II) (32, 33). Like Fe(III), SO₄⁻ can be abiotically oxidized in environments where redox potential fluctuates, creating a sulfur cycle of regenerating terminal electron acceptors to fuel C mineralization (34–36). Both Fe(III) and NO₃⁻ increased CO₂ production, though both decreased cell abundance. Denitrifiers are facultative aerobes that prefer O₂ but have the capacity to switch to NO₃⁻ (or NO₂⁻) reduction under O₂-limited conditions and are phylogenetically diverse (37). Dissimilatory NO₃⁻ reduction to ammonium (DNRA) has also been shown to constitute as much as 75% of the turnover of the NO₃⁻ pool in tropical forest soils (22). The organisms performing DNRA in one instance were discovered to consist of spore-forming bacteria (38). The buildup of NO₂⁻ in the NO₃⁻-amended FACs could have contributed to the repression of activity and cell growth in the NO₃⁻-amended RF-FACs and suggests that while DNRA occurs in the field, it was not likely to be occurring in our FACs.

The addition of different TEAs created changes in C mineralization and populations of methanogens in CF-FACs, where CH₄ production was appreciable in the unamended CF-FACs as well as the CF-FACs amended with Fe(III) and SO₄⁻. While CH₄ was produced in the Fe(III)-amended CF-FAC microcosms, Fe(III) amendment did not increase CO₂ concentrations relative to the background treatment. Iron(III) addition decreased net CH₄ production, likely due to competition with dissimilatory Fe reducers for acetate (21). Net CH₄ production in Fe(III)-amended CF-FACs was significantly depressed, though not as low as in the other FACs, suggesting that either the Fe(III) reducers were not as efficient as the other community members or that the methanogens competing with the Fe(III) reducers for labile C were not as effective under Fe(III)-reducing conditions. The switchgrass-only, Fe(III)-amended, and SO₄⁻-amended CF-FAC microcosms contained 7% *Methanobacterium* species, which are hydrogen- and formate-utilizing methanogens, and [in Fe(III) and switchgrass-only treatments] 3% *Methanosarcina* species, acetate-utilizing methanogens (27, 39, 40). The SO₄⁻-amended CF-FACs did not contain members of *Methanosarcina*, suggesting that SO₄⁻ was somehow inhibitory. Members of the *Clostridia* in clusters 8, 3, and 2 were all significantly enriched in SO₄⁻-amended FACs compared to findings for Fe(III)-amended FACs. There is much phenotypic variation within the *Clostridia* that enables these organisms to live in a variety of environments (41), and apparently even the subtle selective pressures of different additional TEA amendments is enough to select for a specific set of *Clostridia* bacteria.

The established feedstock-adapted consortia generally contained a few dominant species with long tails of richness extending into the hundreds of rare species, which may play a role in stabilizing the activity of these communities. The dominant species, defined as having a relative abundance of 2% or greater, comprised a substantial portion of the total richness of these consortia, which were mostly comprised of the *Clostridia*, as well as members of the *Rhizobiales*, *Bacteroidales*, methanogenic *Archaea*, and *De-*

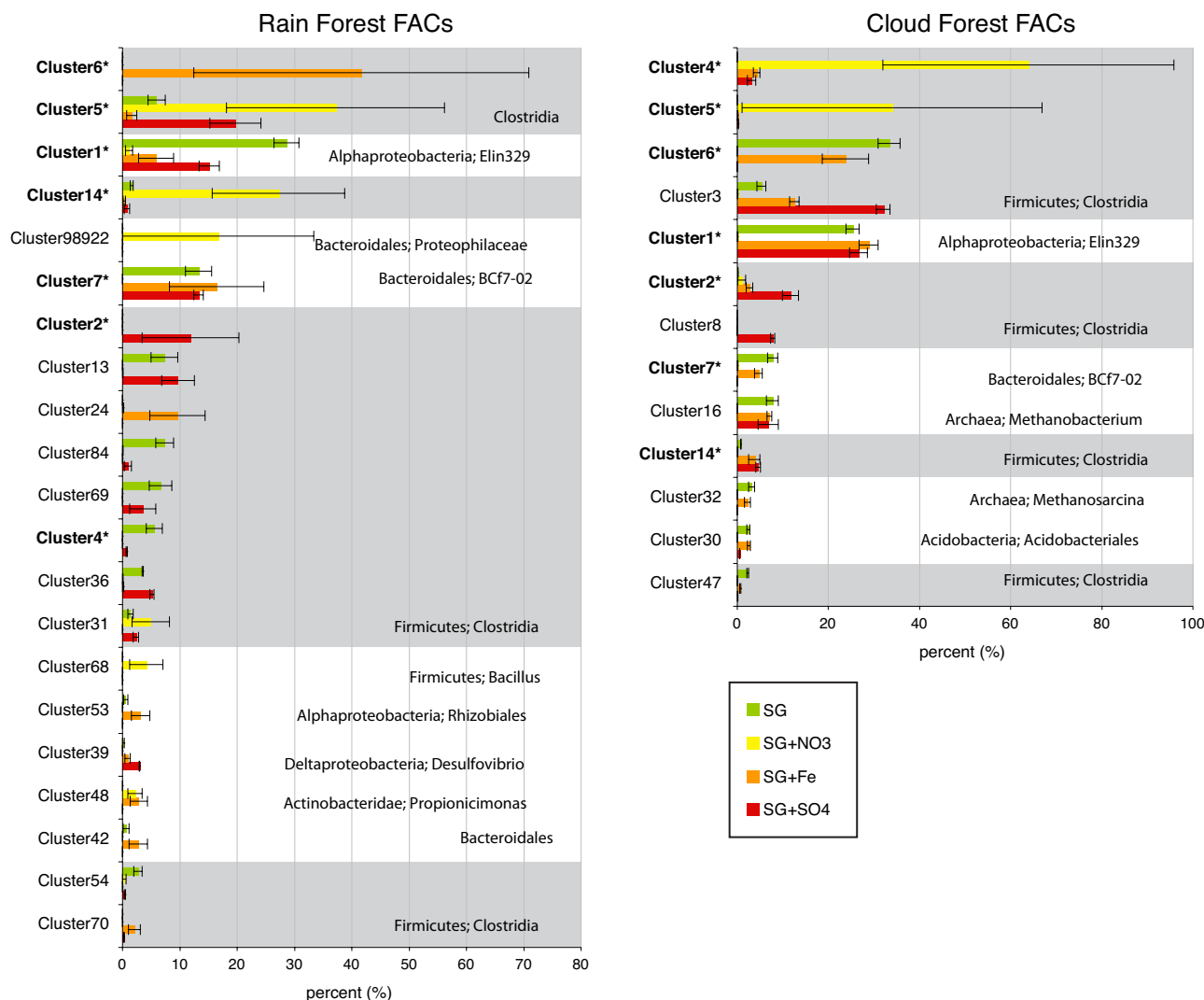


FIG 4 Dominant taxa in the microbial community as determined by pyrosequencing are shown for RF-FACS and CF-FACS based on a relative abundance of 2% or greater. There are 23 taxa in RF-FACS and 14 in CF-FACS that are dominant; taxa are denoted by their cluster number, which was based on 97% SSU rRNA gene sequence identity, and their relative abundance in each TEA treatment is shown as a mean percentage of abundance with standard error ($n = 3$). Bold cluster numbers with an asterisk “*” indicate taxa that are dominant in both the RF- and the CF-FACS.

sulfobivrio. Taxa in the long tail of the communities were made up of taxa from phyla commonly found in soils but uncultured, with little known about their function. In the RF-FACS these included taxa from the phyla *Gemmatimonadetes* and Op10. CF-FACS had the same diversity but fewer dominant taxa and different rare taxa from those of the RF-FACS. The rare taxa have been selected for their ability to grow in the anaerobic enrichments but may also contribute to the activity. For example, it is known that anaerobic cellulose degradation by *Clostridium straminisolvens* CSK1 is stabilized by the presence of noncellulolytic, aerobic bacteria that are thought to reduce the local redox potential (42). Taxa need not be abundant to play important roles in ecosystem function; it has been shown in bog soils that the sulfate reducer *Desulfosporosinus* was present at low (0.006%) abundance but was responsible for the majority of soil SO_4^{2-} reduction (43). Increased richness has been shown to stabilize mixed communities in natural environments, especially when richness is low (below 10 species) (44).

Cooperation among disparate taxa in these mixed communities is possible and will become evident with attempts to isolate contributing members under the same conditions.

MATERIALS AND METHODS

Lab incubations. Soils were collected from the Luquillo Experimental Forest, Puerto Rico, part of the National Science Foundation (NSF)-sponsored Long Term Ecological Research Program. Soils from the Bisley Research Watershed (rain forest site [RF]) and the Pico del Este Short Could Forest (CF) were used as inoculum for three replicate feedstock-adapted consortia (FAC) each. The fieldwork was conducted and samples collected and transported under USDA permit number P526P-08-00634. Site selection was based on redox states of the soil, which change along an elevation and rainfall gradient. The CF site is located in an upper montane tropical cloud forest at approximately 1,050 m above sea level (18°18'N, 65°50'W) and experiences approximately 4 to 5 m rainfall annually and a high frequency of low-redox conditions (45). The RF site is in a lower montane wet tropical forest at approximately 270 m above sea level.

(18°18'N, 65°50'W) and receives approximately 3.5 m of rainfall annually (46). The soils at this site fluctuate between anoxic and oxic (11, 12). Rainfall at both sites is relatively evenly distributed throughout the year; an average annual temperature is 21°C and 23°C at CF and RF, respectively, with little seasonal variation. RF soils are deep, highly weathered, clayey Ultisols, rich in Fe and Al oxides and hydroxides, while CF soils are isomesic Humic Haplaquox, clay rich, wet, and high in organic matter (45, 47).

Soils were collected in January 2009 from the 0-to-10-cm depth and immediately transported fresh to the lab (LBNL, Berkeley CA) under ambient temperature conditions. We weighed out 5 g of fresh soil and added this to deionized water with 0.5 g (dry weight) switchgrass ground to 0.42 mm using a Wiley mill. One control condition consisted of water and soil only, performed in triplicate. Dinitrogen gas was bubbled through all media to remove any dissolved O₂, and containers were quickly sealed with airtight stoppers to maintain anaerobic conditions. The original soil slurry was incubated for 8 weeks and then used to inoculate the FACs at a level of 10% final volume into BMM minimal medium (48) with trace minerals (49, 50) and vitamins (51), buffered to pH 5.5 with morpholineethanesulfonic acid (MES) to match the soil pH. The FACs were transferred two more times after 10 weeks each, maintaining separate lines for each of the three individual replicates; cells were not normalized between transfers. The FAC from round three was split into four treatments with three biological replicates each for a total of 24 FAC cultures. The treatments were baseline switchgrass (15 g liter⁻¹) and baseline switchgrass amended with sodium sulfate (10 mM), potassium nitrate (10 mM), or soluble iron (5 mM). A stock solution of soluble iron was obtained by adding ferric chloride hexahydrate [Fe(III)] to a solution of nitrilotriacetic acid disodium salt and sodium bicarbonate. Dinitrogen gas was bubbled through media to remove any dissolved O₂, and containers were quickly sealed with airtight stoppers to maintain anaerobic conditions. Containers were autoclaved for 20 min at 121°C. These FACs were incubated with treatments for 8 weeks, and then 10% of these cultures were inoculated into fresh media with treatments (as described above). These FACs were each individually continuously monitored for 19 days for net CO₂ and CH₄ accumulation using the Micro-Oxymax respirometer (Columbus Instruments, Columbus, OH). At the end of the experiment, we analyzed FACs for biogeochemistry, enzyme activity, and microbial community structure as detailed below.

Iron analysis. Total iron(II) concentrations were determined by a colorimetric ferrozine assay (52, 53). A 250-μl aliquot was dispensed from each FAC into 5 ml of 0.5 N hydrochloric acid (HCl) and allowed to extract for 20 min. Twenty-five microliters of the HCl solution was then added to 1 ml of ferrozine. Absorbance was immediately recorded at 562 nm on a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc., Brea, CA). A 4-point calibration (0.5, 1, 5, and 15 mM) of iron(II) sulfate was used to quantify sample measurements.

Ion chromatography. Nitrate, nitrite, and sulfate concentrations were measured by ion chromatography (IC) using a model ICS-2000 IC (Dionex Corporation, Sunnyvale, CA) with an IonPac AG11 guard column (4 by 50 mm), IonPac AS11 analytical column (4 by 250 mm), conductivity suppressor (ASRS Ultra), and eluent generator creating a potassium hydroxide gradient. Samples were filtered with a 0.2-μm nylon membrane and diluted 16-fold in ultrapure Milli-Q water. A 3-point calibration (100, 300, and 600 μM) for each anion was used to determine measured concentrations.

Cell counts. Direct counts were made of each sample based on an acridine orange staining technique with epifluorescence counting (54). A sample was taken from each lab incubation, preserved using 4% formaldehyde, and stored at 4°C. Direct counts were obtained by diluting samples 10-fold with filter-sterilized 1× phosphate-buffered saline (PBS) (Sigma Aldrich Corp., St. Louis, MO). Ten microliters of each dilution was placed in a 6-mm microscope slide well and allowed to dry. Wells were stained with 25 mg · ml⁻¹ acridine orange for 2 min in the dark. Unbound acridine orange was rinsed off by dipping slides into deionized water. Cells

were imaged using a fluorescein isothiocyanate (FITC) filter on a Zeiss Axioskop microscope (Carl Zeiss, Inc., Germany). Cell counts are expressed as numbers of cells per ml culture, and fold-increase counts are reported as final counts divided by initial counts.

Enzyme assays. Enzyme activity was determined at the end of the lab incubation by adding 100 μl of culture to wells of a 96-well plate containing 100 μl of the substrates 4-methylumbelliferyl β-d-glucopyranoside (BG) for beta-glucosidase or cellulose activity, β-d-cellobioside-MUB (CBH) for cellobiohydrolase or hemicellulose activity, *N*-acetyl glucosamine-MUB (NAG) for chitinase activity, and β-d-xylopyranoside-MUB (XYL) for xylosidase activity. The fluorescence of each well was determined after a 4-h incubation at 27°C using a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon Inc., Edison, NJ) with 365-nm excitation and 442-nm emission wavelengths. Rates of substrate degradation were calculated as the difference in moles of MUB produced over time based on MUB standards. Enzyme activities for each treatment or replicate were normalized for the initial mass of biomass added and are reported as pmol MUB h⁻¹ g⁻¹ switchgrass.

PLFA. Samples were extracted by the Bligh-Dyer method (55–57). Briefly, 10 ml of a 10:5:4 mixture of methanol-chloroform (pH 7)-phosphate buffer was added to each sample. Fifty microliters of 500-mg/liter 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) was added as an internal standard. The mixture was vortexed, sonicated for 2 min, and extracted at room temperature in the dark for 3 h. Phases were separated with the addition of 2 ml of chloroform and 2 ml of water, followed by centrifugation at 2,000 rpm for 15 min to separate the organic and aqueous layers. The lower organic layer was removed to a clean tube, and 2 ml additional chloroform was added to the original extract, which was vortexed and centrifuged and combined with the first layer. This combined organic layer was dried under N₂. The dried extracts were separated into neutral, glycerol, and phospholipids on a C₁₈ silica column (Sigma Chemical Company, St. Louis, MO) by sequential elution with chloroform, acetone, and methanol. All collected fractions were dried under N₂.

The methanol fractions containing phospholipids were subjected to a mild alkaline hydrolysis to remove the head group and create fatty acid methyl ester (FAME) compounds by resuspending with 1:1 chloroform-methanol and 1 ml of 11.2-mg/liter KOH in methanol. After vortexing for 2 min, they were incubated in a water bath at 37°C for 60 min. The FAME compounds were then neutralized with 200 μl of 0.1 mM acetic acid, extracted three times with 2 ml of hexane, and dried under N₂. Fifty microliters of 46.2-mg/liter methyl undecanoate (Sigma Chemical Company, St. Louis, MO) was added to the dried extracts as an external standard. FAME were detected on an Agilent 6890N gas chromatograph/flame ionization detector (GC/FID) on an HP1 60-m column (0.25-mm inside diameter [ID]) and quantified by comparing to known standards. Peak confirmation was accomplished by using an Agilent 6890 GC/mass spectrometer (MS). Lipid classes were grouped into guilds according to configuration (58–62) as outlined in Table S1 in the supplemental material.

Amplicon pyrosequencing of community SSU rRNA. Samples were taken of liquid medium plus switchgrass from each microcosm, and DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction method as previously described (26). Briefly, frozen samples were added to CTAB extraction buffer and phenol in Lysing Matrix E tubes (Qbiogene) and bead beaten in a FastPrep instrument (Bio101), followed by a chloroform extraction, isopropanol precipitation, and use of the AllPrep DNA/RNA extraction kit (Qiagen). Small subunit (SSU) rRNA gene sequences for high-throughput amplicon pyrosequencing were amplified using the universal primers 926F (5'-aaactYaaaKgaattgacgg-3') and 1392R (5'-acggcggtgtgtRc-3'), where uppercase letters indicate nucleotide redundancies; these primers target the V8 variable region of the 16S rRNA gene from bacteria and archaea as well as the 18S rRNA gene in eukarya (63). The sequences shown do not include adaptor or bar code sequences, and the reverse primer included a 5-bp bar code for multiplexing of samples during sequencing. Emulsion

PCR and sequencing of the PCR amplicons were performed following the manufacturer's instructions for the Roche 454 GS FLX titanium technology, with the exception that the final dilution was $1e-8$. Sequencing tags were analyzed using the software tool PyroTagger (<http://pyrotagger.jgi-psf.org/>), which filters by removing low-quality sequences from the set based on the qual file, trims using a 225-bp sequence length threshold, dereplicates, clusters at the operational taxonomic unit (OTU) level based on 97% identity, and then classifies. Eighteen chimeras were detected and removed, leaving 1,310 taxa detected. Classification was based on the greengenes database of rRNA genes (64) for bacterial and archaeal amplicons and the Silva database for eukaryotic amplicons (65).

Statistical analysis. The experimental design included three biological replicates, four treatments, and two soil inoculum types, for a total of 24 samples. Analyses of variance (ANOVAs) were performed to a statistical significance level of 0.05, and Tukey's honestly significant difference (HSD) test was used to determine treatments with statistically indistinguishable measurements. Ordination of the whole community detected by pyrosequencing was performed using nonmetric multidimensional scaling with the Bray-Curtis distance measure based on unifracs distances (66, 67). Joint plots are vectors indicating the direction and magnitude of correlation to individual samples; joint plots were calculated for all measured variables against the community ordination, and only the factors with significant ($P < 0.05$) correlations are shown. In all results, means and standard errors of the mean are reported for 3 biological replicates unless otherwise indicated. A multiresponse permutation procedure (MRPP) was used to determine differences in inoculum or TEA amendment treatment within the microbial community distance matrix. This test yields metric A, which describes the within-group homogeneity and can range from 0 if the groups are completely different to 1 if the groups are identical; for ecological data, it is uncommon to observe A values greater than 0.3 (68). We used Benjamini-Hochburg-adjusted P values in PLFA peak analysis (69). To calculate richness or diversity (by Shannon's H or Simpson's D index), data from CF- and RF-FACs were pooled after determining that they were not significantly different.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00249-11/-/DCSupplemental>.

FIG S1, PDF file, 0.1 MB.
FIG S2, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.1 MB.
Table S5, PDF file, 0.1 MB.
Table S6, PDF file, 0.1 MB.

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